Supporting Information

Chemical induction of Hsp70 reduces α-synuclein aggregation in neuroglioma cells

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Supporting Information includes:
Supplementary Figures S1-S6

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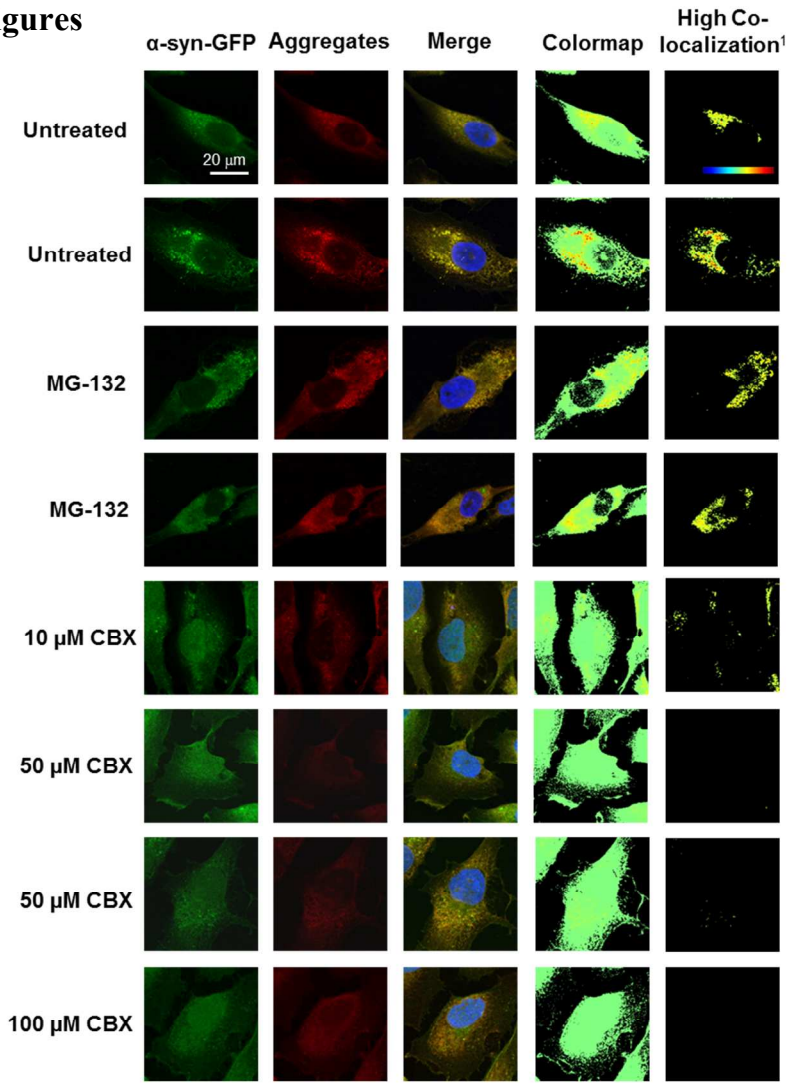
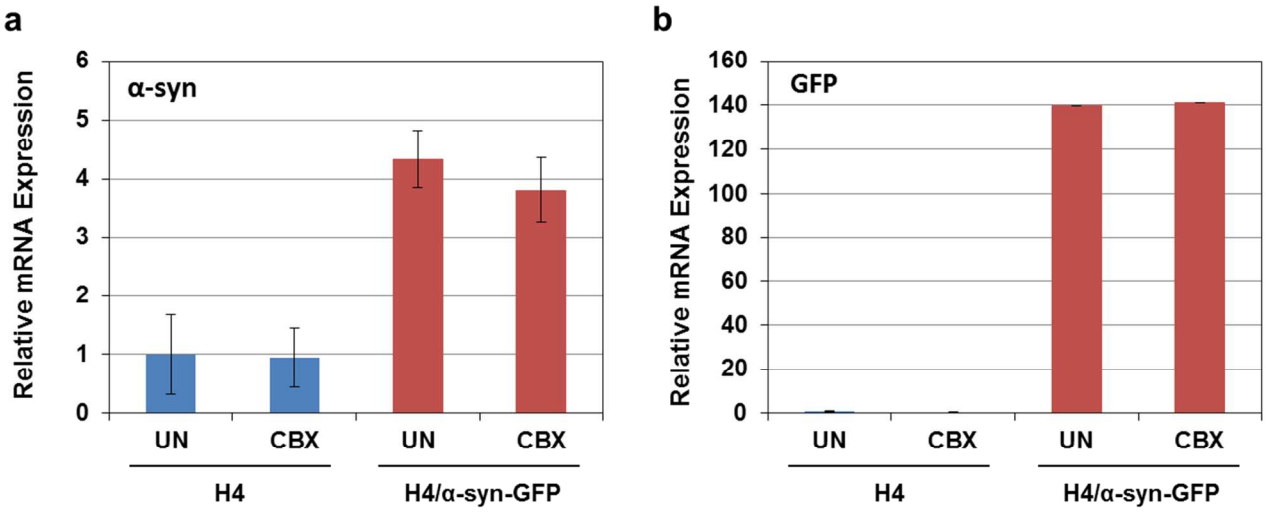
Supplementary Methods

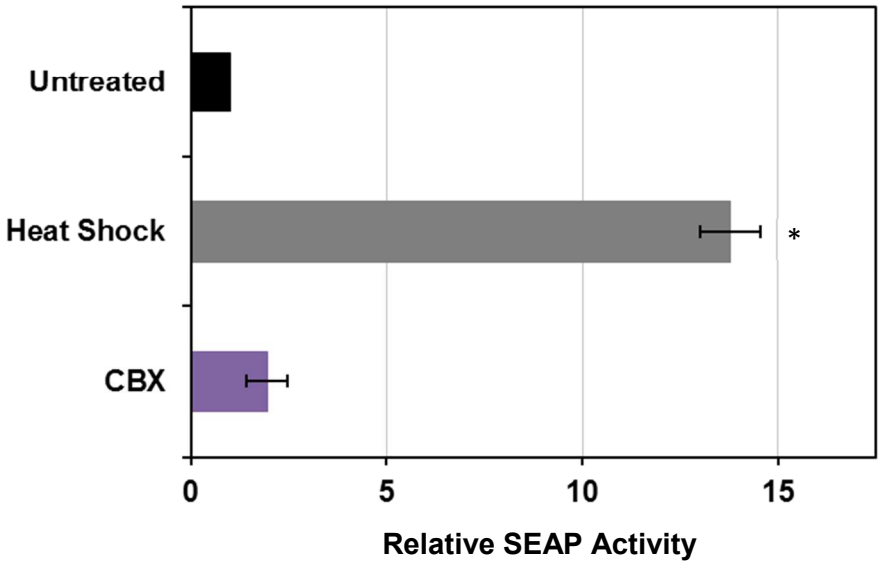
Supplementary Figures



Supplementary Figure S1. CBX decreases α-syn-containing aggregates in H4/α-syn-GFP cells (Related to Figure 1). H4/α-syn-GFP cells untreated or treated with MG-132 (0.5 µM) or CBX (10, 50, or 100 µM) for 16 h were analyzed by fluorescence microscopy. Images of α-syn-GFP fluorescence (green, column 1) and aggregates, detected using the ProteoStat® dye (red, column 2), were merged (column 3) and analyzed using NIH ImageJ software. Colocalization of α-syn-GFP and ProteoStat® dye was evaluated using the Colocalization Colormap plugin (column 4): "hot" colors represent a positive correlation and "cold" colors represent a negative correlation. High colocalization represented by hot colors was depicted by filtering colormap images based on hue as described in the Methods (pixels 1-60) (column 5). Scale bar represents 20 µm. Multiple images corresponding to the most relevant culturing conditions (MG-132 0.5 µM and CBX 50 µM) are reported.

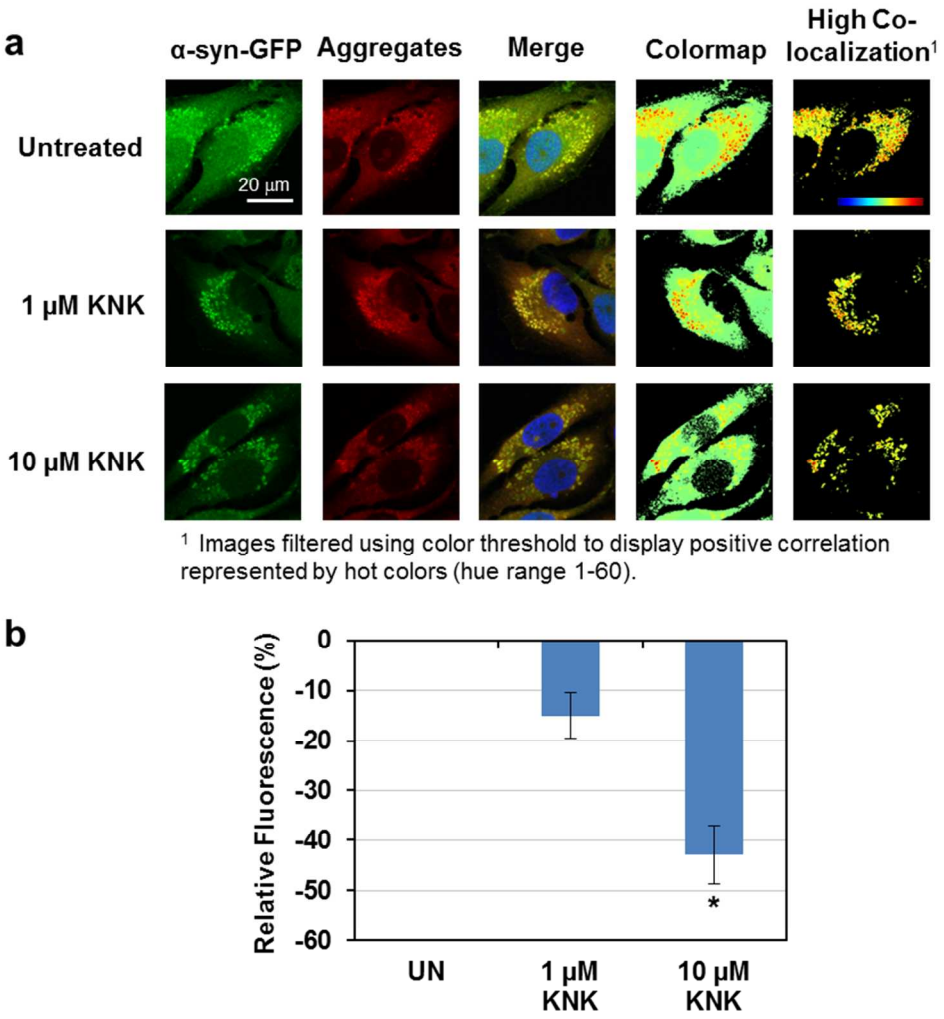


Supplementary Figure S2. CBX does not alter the expression of α-syn or GFP in H4 and H4/α-syn-GFP cells (Related to Figure 1). Relative mRNA expression of **a)** α-syn and **b)** GFP in H4 and H4/α-syn-GFP cells treated with CBX (50 μM) for 16 h. mRNA expression levels were evaluated by quantitative RT-PCR, corrected for the expression of the housekeeping gene, GAPDH, and normalized to those of untreated cells.

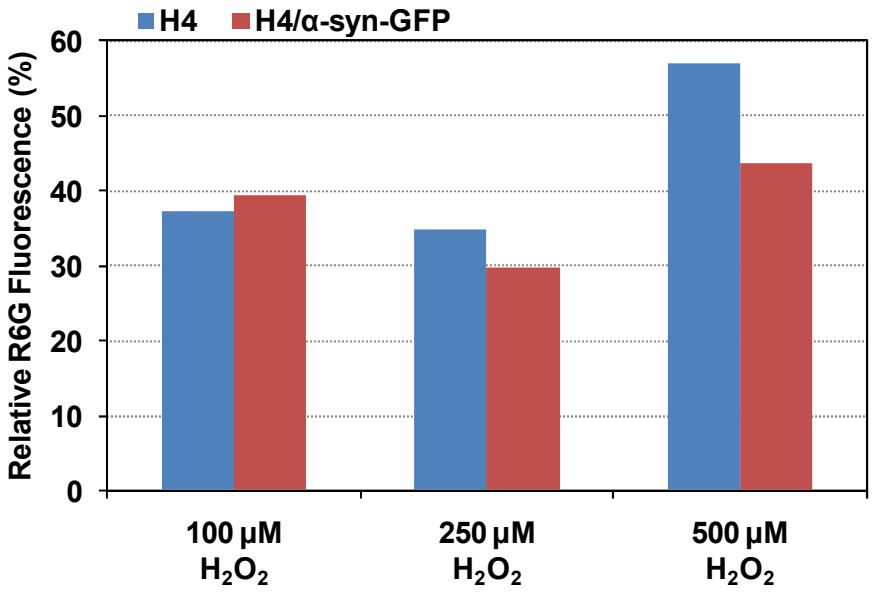


Supplementary Figure S3. CBX mildly upregulates Hsp70 in H4 cells. (Related to Figure 2)

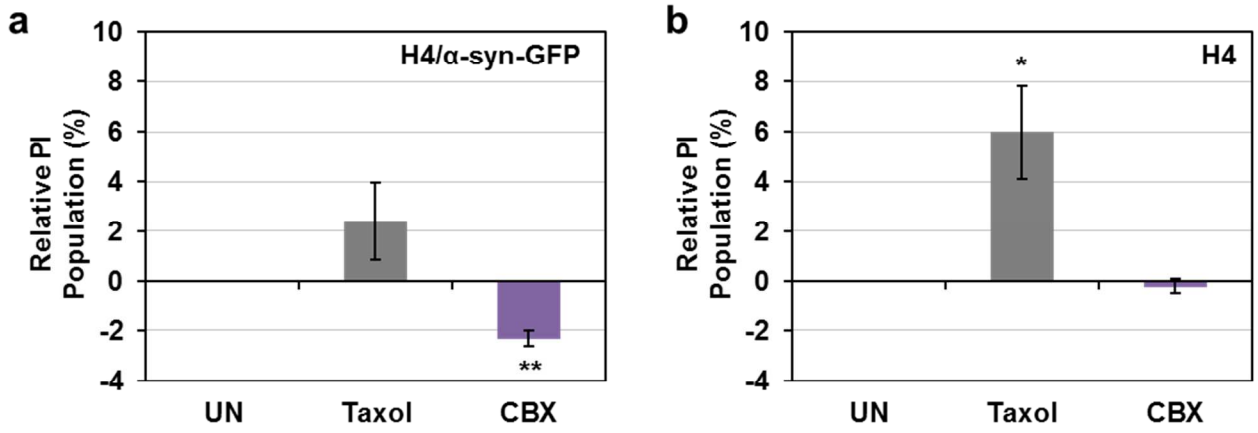
SEAP reporter assay using an expression vector that contains the promoter region of human Hsp70 gene fused to the gene encoding secreted embryonic alkaline phosphatase in H4 cells untreated or subjected to heat shock (42°C for 2 h followed by 4 h incubation at 37°C) or treated with CBX (50 μM) (*$p < 0.05$). SEAP activity was evaluated by measuring the absorbance of QUANTI-Blue reagent and normalized to untreated H4 cells. The experiments were repeated three times and data are reported as the mean ± SD.



Supplementary Figure S4. Inhibition of HSF1 enhances α-syn aggregation and decreases α-syn solubility in H4/α-syn-GFP cells (Related to Figure 5). a) α-syn aggregation in H4/α-syn-GFP cells treated with the HSF1 inhibitor KNK-437. H4/α-syn-GFP cells untreated or treated with KNK-437 (1 or 10 μM) for 16 h were analyzed by fluorescence microscopy. Images of α-syn-GFP fluorescence (green, column 1) and aggregates, detected using the ProteoStat® dye (red, column 2), were merged (column 3). Scale bar represents 20 μm. **b)** α-syn solubility in H4 cells treated with the HSF1 inhibitor KNK-437. H4 cells were transfected to express the αsyn-split GFP system and treated with KNK-437 (1 or 10 μM) for 16 h. Fluorescence complementation was evaluated by measuring GFP fluorescence by flow cytometry. Relative fluorescence was calculated by normalizing the fluorescence of treated cells to that of untreated cells. The data are reported as the mean ± SD (n=3).



Supplementary Figure S5. Oxidative stress induced by H_2O_2 in H4 and H4/α-syn-GFP cells. (Related to Figure 6a and b). ROS generation in H4 and H4/α-syn-GFP cells treated with H_2O_2 (100, 250, and 500 μM) for 1 h was quantified by measuring rhodamine 6G fluorescence by flow cytometry.



Supplementary Figure S6. CBX does not induce apoptosis in H4 and H4/α-syn-GFP cells. (Related to Figure 6c and d). Relative PI population in **a)** H4/α-syn-GFP and **b)** H4 cells treated with taxol (50 nM) and CBX (50 μM) for 16 h (*$p < 0.05$, **$p < 0.01$). The experiments were repeated three times and the data are reported as the mean ± SD.

Supplementary Tables

Supplementary Table S1. Quantitative analysis of total cellular aggregation in H4 and H4/α-syn-GFP cells treated with CBX. (Related to Figure 1 and Table 1).

ProteoStat® Binding (total cellular aggregation)		
Cell Treatment	H4[a]	H4/α-syn-GFP[a]
Untreated	12.5 ± 2.9	16.3 ± 3.5
MG-132	15.0 ± 4.4	14.1 ± 2.7
CBX*	20.3 ± 2.7	7.1 ± 1.7

[a] Average pixel intensity of ProteoStat® dye in images of H4 and H4/α-syn-GFP cells untreated and treated with MG-132 (0.5 μM) or CBX (50 μM) using ImageJ (*$p < 0.005$). The experiments were repeated three times and the data are reported as the mean ± SD.

Supplementary Table S2. α-syn assembly PCR primers. (Related to Methods)

Primer Name	Sequence (5' – 3')
α-syn_1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGGATGTATTCATGAAAGGACT
α-syn_2	CAGCAGCCACAACTCCCTCCTTGGCCTTTGAAAGTCCTTTCATGAATACATCCATGGTTCT
α-syn_3	AGGAGGGAGTTGTGGCTGCTGCTGAGAAAACCAAACAGGGTGTGGCAGAAGCAGCAGGAAAG
α-syn_4	CCTCCTTGGTTTTGGAGCCTACATAGAGAACACCCTCTTTTGTCTTTCCTGCTGCTTCTGCCACA
α-syn_5	TATGTAGGCTCCAAAACCAAGGAGGGAGTGGTGCATGGTGTGGCAACAGTGGCTGAGAAGACC
α-syn_6	CGTCACCACTGCTCCTCCAACATTTGTCACTTGCTCTTTGGTCTTCTCAGCCACTGTTGCCA
α-syn_7	TGTTGGAGGAGCAGTGGTGACGGGTGTGACAGCAGTAGCCCAGAAGACAGTGGAGGGAGCAG
α-syn_8	CAACTGGTCCTTTTTGACAAAGCCAGTGGCTGCTGCAATGCTCCCTGCTCCCTCCACTGTCTTCTG
α-syn_9	CTGGCTTTGTCAAAAAGGACCAGTTGGGCAAGAATGAAGAAGGAGCCCCACAGGAAGGAATTC
α-syn_10	GCATTTCATAAGCCTCATTGTCAGGATCCACAGGCATATCTTCCAGAATTCCTTCCTGTGGGGCTCCT
α-syn_11	GATCCTGACAATGAGGCTTATGAAATGCCTTCTGAGGAAGGGTATCAAGACTACGAACCTGAAGC
α-syn_12	GGGGACCACTTTGTACAAGAAAGCTGGGTCGGCTTCAGGTTCGTAGTCTTGATACC

Supplementary Table S3. Primer Sequences Used in Quantitative RT-PCR *(1)*. (Related to Methods)

Gene	GenBank Accession Code	Forward Primer	Reverse Primer
GAPDH	NM_002046	5'-GTCGGAGTCAACGGATT-3'	5'-AAGCTTCCCGTTCTCAG-3'
Hsp27	X54079	5'-AAGTTTCCTCCTCCCTGTCC-3'	5'-CGGGCTAAGGCTTTACTTGG-3'
Hdj1	NM_006145	5'-CGCCGAGGAGAAGTTC-3'	5'-CATCAATGTCCATGCCTT-3'
Hsp70	NM_005345	5'-GGAGGCGGAGAAGTACA-3'	5'-GCTGATGATGGGGTTACA-3'
Hsp90	NM_005348	5'-GATAAACCCTGACCATTCC-3'	5'-AAGACAGGAGCGCAGTTTCATAAA-3'

Supplementary Methods

Primers and Plasmids. The cDNA encoding human wild type α-syn (P37840) was generated by assembly PCR using primers reported in Supplementary Table S2. The PCR product was first cloned into pENTR™11 and then transferred into pcDNA™6.2/C-EmGFP-DEST using Gateway® recombination cloning technology (Invitrogen) according to the manufacture's protocol.

Cell Lines and Stable Transfections. Human H4 neuroglioma cells (HTB-148, ATCC) were cultured in high glucose DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 1% PSQ, 4 mM L-Glutamine, and 1mM sodium pyruvate, and maintained at 37 °C and 5% CO_2. Cells were plated 24 h before transfection in medium without PSQ and transfected with pcDNA6.2/α-syn-EmGFP using Lipofectamine2000 according to the manufacturer's instructions (Invitrogen). After 16 h, the transfection medium was replaced with fresh, complete medium and cells were incubated for an additional 24 h. Stably transfected cells were selected by subculturing cells to a concentration of 2.5 x 10^4 cells/mL in complete medium with 5 μg/mL Blasticidin S HCl. Blasticidin-resistant cells were plated in 96-well plates at a concentration of 0.5 cells/well to isolate monoclonal populations. GFP fluorescence was detected by microscopy (BC-364 Inverted Epifluorescent Microscope, Jenco) and by flow cytometry (FACSCanto™II, BD Biosciences).

Aggregation Studies. H4 and H4/α-syn-GFP cells were treated with MG-132 (0.5 μM) or CBX (50 μM) for 16 h at 37 °C. α-syn-GFP aggregation was measured using the ProteoStat® Aggregation detection kit (Enzo Life Sciences) according to manufacturer's protocol.

Protein aggregation in H4/α-syn-GFP cells was detected by fluorescence microscopy. Colocalization of α-syn-GFP and the ProteoStat® dye in H4/α-syn-GFP cells was evaluated using the *Colocalization Colormap* script, an ImageJ plugin that calculates the correlation of intensity between complementary fluorescent signals. The results are presented as a colormap where hot colors represent positive correlation and cold colors represent negative correlation *(2)*. Colormaps were analyzed using the ImageJ plugin, Threshold Colour, which allows RGB images to be filtered based on hue, saturation, and brightness (http://www.dentistry.bham.ac.uk/landinig/software/software.html). To indicate high colocalization, the hue was filtered to display pixel intensities from 0 to 35 and designated as red pixels. To indicate low colocalization, designated as yellow pixels, the hue was filtered to display pixel intensities from 35 to 60. Pixels in the hue range from 60 to 255 were considered negative correlation and were not evaluated in this study.

To quantify aggregation in H4 and H4/α-syn-GFP cells, the average pixel intensity of images from cells stained with the ProteoStat® dye was evaluated by determining the brightness of each pixel on a scale of 0 to 255 where 0 is black and 255 is white and calculating the average pixel brightness across the entire image.

H4 and H4/α-syn-GFP cells were treated with small molecules as described above and the aggregation propensity factor (APF) was calculated using the following formula: $APF = 100 \times (MFI_{treated} - MFI_{control})/MFI_{treated}$ where MFI is the mean fluorescence intensity of the ProteoStat® dye and untreated cells were used as the control. Fluorescence intensity was measured by flow cytometry (FACSCanto™ II, BD Biosciences) using a 488-nm argon laser.

Cell Fractionation. Detergent solubility and cell fractionation was conducted as previously described *(3)*. H4/α-syn-GFP cells were plated in 10-cm culture dishes at concentration of 1.0×10^5 cells/mL and treated with small molecules for 24 h. The soluble protein fraction was extracted by incubating the cells in

Complete Lysis-M Buffer (Roche) supplemented with 1% Triton X-100 on ice with gentle agitation for 30 min followed by centrifugation at 15,000 x g for 60 min at 4 °C. The pellet was resuspended in Complete Lysis-M Buffer supplemented with 2% SDS and 8M urea and sonicated to collect the insoluble protein fraction. Protein concentrations were determined by Bradford assay, and samples were diluted to the same concentration and separated by 12% SDS-PAGE. Western blot analyses were performed using mouse anti-α-syn (Sigma), chicken anti-GFP (Anaspec), and rabbit anti-GAPDH (Santa Cruz) antibodies and appropriate secondary antibodies (HRP conjugated anti-mouse (Stressgen), anti-chicken (Santa Cruz), and anti-rabbit (Santa Cruz)). Blots were visualized using Luminata™ Forte Western HRP Substrate (Millipore) and bands were quantified with NIH ImageJ software.

Hsp70 Western blots. H4/α-syn-GFP cells were plated in 10-cm culture dishes at a concentration of 1.0 x 10^5 cells/mL and subjected to heat shock (42°C for 2 h followed by 4 h incubation at 37°C) or treated with small molecules for 24 h. Protein was extracted by incubating the cells in TNT buffer (50mM Tris pH 7.2, 150 mM NaCl, 1% Triton X-100) on ice with gentle agitation for 30 min followed by centrifugation at 15,000 x g for 15 min at 4 °C. Total protein of each lysate was determined using BCA Protein Assay Reagent (Thermo Scientific, Pierce). Equal protein amounts, totaling 10 µg for each treatment, were separated using a 10% polyacrylamide gel. The proteins were transferred onto nitrocellulose using a Pierce Fast Semi-Dry blotter at 12V for 25 min. The nitrocellulose membrane was blocked using TBST containing 5% non-fat dry milk for 1 h at room temperature. The nitrocellulose was then cut at the 55 kDa marker and the top half was incubated with mouse anti-Hsp70 (StressMarq, SMC113A) at a dilution of 1:1000, while the bottom half was incubated with mouse anti-Hdj1 (StressMarq, SMC145C) at a dilution of 1:2000 overnight with rocking at 4 °C. The following day the membranes were washed 3 times for 10 min with TBST followed by a 2 hour incubation with anti-mouse IgG, HRP-linked secondary antibody (Cell Signaling, 7076) at a dilution of 1:5000 at room temperature. The membranes were then washed 3 times 10 min with TBST, followed by a 5 min exposure to

SuperSignal WestPico Chemiluminescent substrate (Thermo Scientific, Pierce), imaged using a Kodak Image Station 440CF (Eastman Kodak Co.), and quantified using ImageJ version 1.47m software (NIH). The data represent the means +/- standard deviation of n=3 technical replicates from a representative experiment. Equal loading was visualized by stripping the lower membrane of bound antibodies for 1 h with 0.1M glycine pH 2.3, followed by re-blocking and probing with rabbit anti-beta actin (Cell Signaling, 4967) at a dilution of 1:2500 with rocking overnight at 4 °C. After following the same washing procedure described above, the blot was incubated with anti-rabbit IgG, HRP-linked (Cell Signaling, 7074) at a dilution of 1:5000 for 2 h at room temperature and processed for imaging as described above.

Quantitative RT-PCR. RT-PCR was conducted as previously described *(4)*. Total RNA was extracted using RNAGEM™ Tissue reagent (ZyGEM). cDNA was synthesized from total RNA using qScript™ cDNA SuperMix (Quanta Biosciences) and quantified using a NanoDrop (Thermo Scientific). Quantitative PCR reactions were performed using PerfeCTa™ SYBR Green FastMix (Quanta Biosciences) in a CFX96 Real-Time PCR Detection System (Bio-Rad) with corresponding primers in Supplementary Table S3 online. Samples were heated for 2 min at 95°C and amplified using 45 cycles of 1 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Analyses were conducted using CFX Manager software (Bio-Rad) and the threshold cycle (C_T) was extracted from the PCR amplification plot. The ΔC_T value was used to describe the difference between the C_T of a target gene and the C_T of the housekeeping gene, GAPDH: $\Delta C_T = C_T$ (target gene) - C_T (GAPDH). The relative Hsp70 mRNA expression level of treated cells was normalized to that of untreated cells: relative mRNA expression level = 2 exp [-(ΔC_T (treated cells) – ΔC_T (untreated cells))]. Each data point was evaluated in triplicate and measured three times.

SEAP Reporter Assay. Activation of the promoter region of human Hsp70 was monitored by measuring the accumulation of secreted embryonic alkaline phosphatase (SEAP) in the medium of H4 and H4/α-

syn-GFP cells transfected with pDRIVE5SEAP-hHSP70 (Invivogen) and treated with CBX. H4 and H4/α-syn-GFP cells were plated in 24-well plates at 1 x 10^5 cells/mL and grown for 16 h. Cells were transfected with 0.3 μg of pDRIVE5SEAP-hHSP70 using Lipofectamine 2000 (Invitrogen). After 16 h, the transfection medium was replaced with fresh medium and cells were treated with CBX (100 μM), or heat shock (42°C for 2 h followed by 4 h incubation at 37°C). The medium was collected and centrifuged for 5 min at 10,000 rpm. SEAP activity was analyzed using QUANTI-Blue according to manufacturer's instructions (Invivogen) and light emission was measured at OD_{620} using a microplate reader (BioTek Synergy HT).

Immunofluorescence Studies. Cells were cultured on poly L-lysine (Sigma) coated glass coverslips. After treatment with small molecules or heat shock for the specified amount of time, cells were fixed using 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with 8% bovine serum albumin. Next, cells were incubated with rabbit HSF1 antibody (Enzo Life Sciences) for 1 h, washed with 0.1% Tween/PBS, and incubated for 1 h with DyLight 549 goat anti rabbit antibody (Rockland Immunochemical). Images were collected at 100X using a confocal microscope (FluoView FV1000, Olympus) and analyzed using NIH ImageJ software.

Split GFP Assay. The split GFP assay was conducted as previously described *(5)*. H4 cells were plated in 6-well plates and incubated for 24 h at 37°C. The media was removed and replaced with fresh media containing 0.33 μg of pCMV-mGFP/αsyn-GFP_{11}, and 0.67 μg of pcDNA4/TO/GFP_{1-10} per well and transfected using Lipofectamine 2000 (Invitrogen). Transfection reactions were incubated for 16 h and fluorescence was measured using a flow cytometer (FACSCanto™ II, BD Biosciences).

Toxicity Assay. Induction of apoptosis was measured as previously described *(6, 7)*. H4 and H4/α-syn-GFP cells were treated with taxol (50 nM) and CBX (50 μM) for 16 h at 37 °C. Cells were collected and resuspended in 100 μL of 1X binding buffer (BD Biosciences). Cell toxicity was tested by incubating samples with 5 μL of Annexin V-Cy5 (BD Biosciences) for 20 min in the dark at room temperature. Samples were diluted with 400 μL 1X binding buffer and analyzed by flow cytometry (FACSCanto™ II, BD Biosciences) with a 533-nm Helium Neon laser for Cy5 fluorescence.

Measurement of intracellular ROS generation. Dihydrorohodamine 6G (DHR6G) was used to measure oxidative stress as previously described *(8)*. Cells were cultured in medium containing MG-132 (0.5 μM) or CBX (50 μM) for 16 h at 37 °C. Samples treated with 100 μM hydrogen peroxide (H_2O_2) for 1 h at 37 °C were used as positive control. Cells were washed with PBS and incubated with 5 μM DHR6G (Anaspec) in serum-free DMEM for 30 min at 37 °C. Cells were collected in PBS, centrifuged at 300 x g for 5 min, and washed with PBS. DHR6G fluorescence was analyzed by flow cytometry using a 488-nm argon laser and 585/42 band pass filter.

Statistical Analysis. All data are presented as the mean ± SD, and statistical significance was calculated using a two-tailed t-test.

Supplementary References

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